Aldosterone Potentiates Angiotensin II–Induced Signaling in Vascular Smooth Muscle Cells

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Background—In a double-transgenic human renin and human angiotensinogen rat model, we found that mineralocorticoid receptor (MR) blockade ameliorated angiotensin II (Ang II)–induced renal and cardiac damage. How Ang II and aldosterone (Ald) might interact is ill defined.

Methods and Results—We investigated the effects of Ang II (10⁻² mol/L) and Ald (10⁻⁷ mol/L) on extracellular signal–regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling in vascular smooth muscle cells (VSMCs) with Western blotting and confocal microscopy. Ang II induced ERK 1/2 and JNK phosphorylation by 2 minutes. Ald achieved the same at 10 minutes. Ang II + Ald had a potentiating effect by 2 minutes. Two oxygen radical scavengers and the epidermal growth factor receptor (EGFR) antagonist AG1478 reduced Ang II–, Ald–, and combination-induced ERK1/2 phosphorylation. Preincubating the cells with the MR blocker spironolactone (10⁻⁶ mol/L) abolished Ang II–induced ROS generation, EGFR transactivation, and ERK1/2 phosphorylation.

Conclusions—Ald potentiates Ang II–induced ERK-1/2 and JNK phosphorylation. Oxygen radicals, the MR, and the EGFR play a role in early signaling induced by Ang II and Ald in VSMCs. These in vitro data may help explain the effects of MR blockade on Ang II–induced end-organ damage in vivo. (Circulation. 2004;109:2792-2800.)

Key Words: angiotensin ■ aldosterone ■ receptors ■ kinases ■ reactive oxygen species

The Randomized Aldactone Evaluation Study (RALES) showed that adding spironolactone (Spi) to an ACE inhibitor and furosemide-based heart failure treatment reduced mortality by 30%.1 The beneficial effects of Spi were reproduced mortality by 30%.1 The beneficial effects of Spi were

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000131860.80444.AB

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light ether anesthesia. Three measurements per heart were averaged. Rats were killed at the age of 7 weeks.

Cell Culture
Aortic VSMCs were isolated from SD rats.2,12 Passages 2 to 4 were used for immunohistochemistry and passage 4 to 10 for Western blotting. VSMCs were phenotyped by staining for muscle-specific α-actin (Dako) and desmin (Boehringer-Mannheim). VSMCs were also analyzed for MR expression. TaqMan polymerase chain reaction (PCR) demonstrated RNA expression of the receptor (primer sequences as follows: MR-F, GCACCTACACATCCGCCG; MR-R, TCCTAGGCTCGACACCGTC; MR-P, FAM-CCATGATCT-GGAGAACATCGACG-TAMRA; data not shown). Cells were treated with Ang II (Sigma), Ald (Clinalfa), glutathione (GSH) (Sigma), or Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid; Sigma). The following blockers were used as indicated: AG 1478 (Calbiochem) and Spi (Sigma). All experiments were performed under 18-hour serum-free conditions.

Immunohistochemistry
Confocal microscopy was performed as described.12 At least 50 to 80 cells from each experiment were examined under each condition by 2 different investigators without knowledge of the origin of the specimens. Quantification was performed with histogram function in the MRC Laser Sharp software. The subcellular regions were outlined manually, and the calculated mean fluorescence intensity was obtained for the delineated regions. Data are presented as the mean fluorescence intensity in the respective cell area. Immunohistochemistry for collagen IV and phospho-ERK was performed.10,11 For collagen IV detection, we used an antibody from Southern Biotech (1:500), and for phospho-ERK, from Santa Cruz (1:100). Western blotting. VSMCs were phenotyped by staining for muscle-specific α-actin (Dako) and desmin (Boehringer-Mannheim). VSMCs were also analyzed for MR expression. TaqMan polymerase chain reaction (PCR) demonstrated RNA expression of the receptor (primer sequences as follows: MR-F, GCACCTACACATCCGCCG; MR-R, TCCTAGGCTCGACACCGTC; MR-P, FAM-CCATGATCT-GGAGAACATCGACG-TAMRA; data not shown). Cells were treated with Ang II (Sigma), Ald (Clinalfa), glutathione (GSH) (Sigma), or Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid; Sigma). The following blockers were used as indicated: AG 1478 (Calbiochem) and Spi (Sigma). All experiments were performed under 18-hour serum-free conditions.

Western Blot
The following primary antibodies were used: polyclonal ERK1/2 (NEB; 1:1000), phospho-ERK1/2 (NEB; 1:1000), phospho-ELK-1 (NEB; 1:1000), phospho-JNK (Dianova; 1:1000), and p-EGFR (NEB; 1:1000). Peroxidase-conjugated secondary antibodies were from Sigma (1:5000). Blots were developed with the chemiluminescence substrate and visualized on Kodak films. Three to 5 cell stimulation experiments of each protocol were performed and quantified. For semiquantification, the most intense band was defined as 100%. All other bands of the experiment were calculated as percentage of the maximum.

p42/44 MAP Kinase Assay
The kinase assay for ERK 1/2 (p42/44 kinase assay) was performed with a kit from New England BioLabs. Briefly, after stimulation, active mitogen-activated protein (MAP) kinase was selectively immunoprecipitated. The precipitate was incubated with ATP and Elk-1 fusion protein in a kinase buffer. This allows active MAP kinase to phosphorylate Elk-1. Elk-1 phosphorylation was measured by Western blotting and quantified as described above.

Dichlorofluorescein to Measure Intracellular Reactive Oxygen Species
Intracellular reactive oxygen species (ROS) production was measured in rat VSMCs by the method of Ohba et al.13 Briefly, cells were kept in serum-free conditions for 24 hours (0.1% BSA). Cells were preincubated with Spi 10−6mol/L for 30 minutes or DMSO 10 μmol/L for 30 minutes. H2DCF-DA (2′,7′-dichlorofluorescein-diacetate, Sigma, 5 μmol/L) was added, and cells were stimulated with Ang II 10−7mol/L.

Statistics
Data are presented as mean±SEM. Statistical significance was tested by ANOVA, blood pressure and albuminuria by repeated-measures ANOVA and the Scheffé test, and ROS generation by SPSS. We used a general linear model with repeated measurements and post hoc by paired t test with the Bonferroni correction. A value of P<0.05 was considered statistically significant. The data were analyzed by use of StatView statistical software.

Results
MR Blockade Prevents Ang II–Induced End-Organ Damage In Vivo
Untreated dTGR (n=14) showed increased systolic blood pressure compared with Epl-treated dTGR (n=14) and nontransgenic (n=7) rats (204±5 versus 180±5 versus 119±6 mm Hg, P<0.05, respectively). Systolic blood pressure at week 7 was 61 mm Hg higher in Epl-treated dTGR compared with SD rats (P<0.05; Figure 1A). Urinary albumin excretion was markedly higher in dTGR than in SD rats: 17.8±2.1 versus 0.2±0.02 mg/d (P<0.001). Epl treatment reduced albuminuria (9.2±1.3 mg/d; P<0.05; Figure 1B). Epl also reduced collagen IV matrix deposition in the kidney (n=5 each; Figure 1C) and heart (data not shown). Untreated dTGR show ERK1/2 phosphorylation in the media of renal dTGR vessels, which was reduced by Epl (n=5 each; Figure 1D). Epl also reduced cardiac hypertrophy index (4.5±0.1 versus 5.4±0.2 mg/g; P<0.05; Figure 1E) and improved left ventricular diastolic function (normalized E/A ratio; Figure 1F) compared with untreated dTGR. Nevertheless, cardiac hypertrophy index remained increased in Epl-treated dTGR compared with nontransgenic SD rats (3.6±0.1 mg/g; P<0.05). These data document marked amelioration of renal and cardiac damage by MR blockade.

Ald Potentiates Ang II–Induced ERK Phosphorylation
Ang II (10−7 mol/L) induced ERK phosphorylation in VSMCs with a maximal intensity after 2 minutes (Figure 2A). After Ald (10−7 mol/L), the maximal intensity of ERK phosphorylation was observed at 10 minutes (Figure 2B). The combination of Ang II and Ald resulted in a stronger ERK phosphorylation at 1 and 2 minutes than with Ang II or Ald alone (Figure 2, C and E). Western blot and confocal microscopy experiments showed that Ang II and Ald at a lower concentration (both 10−9 mol/L) still caused a similar potentiation (data not shown). Using the ERK1/2 MAP kinase assay, kinase activity was increased after Ang II and Ald alone as well as after the combination. However, the combination resulted in a higher MAP kinase activity, resulting in enhanced Elk-1 phosphorylation compared with the single compounds (Figure 2D). We also investigated Ang II– and/or Ald-induced ERK phosphorylation in the presence of the protein synthesis inhibitors actinomycin D and cycloheximide. Neither inhibitor affected short-term ERK phosphorylation, supporting a nongenomic Ang II/Ald effect (data not shown).

Ald Potentiates Ang II–Induced JNK Phosphorylation
Ang II (10−7 mol/L) also induced JNK phosphorylation with a maximal intensity after 2 minutes, whereas the maximal intensity of JNK phosphorylation after Ald (10−7 mol/L) stimulation was observed at 10 minutes. When the cells were
stimulated with the combination of both Ang II and Ald, a significantly stronger JNK phosphorylation was observed at 1 minute than with the single compounds alone (data not shown).

Ang II and Ald Signaling Is Mediated Through Oxygen Radicals
VSMCs were preincubated with GSH for 90 minutes before stimulation. GSH (2 mmol/L) preincubation suppressed both
ERK (Figure 3, A through C) and JNK (Figure 3, D through F) phosphorylation after stimulation with Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and the combination of Ang II and Ald. To verify the effect, we preincubated the cells with a distinct oxygen radical scavenger, Tiron. Tiron preincubation (10 μmol/L) caused a similar suppression of ERK phosphorylation induced by Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and the combination of Ang II and Ald (data not shown).

Effect of Spi on Ang II Signaling
Next, we preincubated VSMCs for 30 minutes with Spi. Thereafter, the cells were stimulated with Ang II (10⁻⁷ mol/L) for 10 minutes. Spi decreased Ang II–induced ERK phosphorylation at 10 minutes but not at 2 minutes, as shown by Western blot (Figure 4A) and by confocal microscopy (Figure 4B). Consistent with this result, Ang II–induced EGFR phosphorylation was reduced by Spi at 10 minutes (Figure 4B; *P=0.01); no effect was observed at 2 minutes. Furthermore, we measured the effect of Spi on ROS production induced by Ang II (10⁻⁷ mol/L) and Ald (10⁻⁷ mol/L). Spi reduced Ang II–induced ROS generation from 5 minutes onward (Figure 4C, *P=0.05 at 5 minutes, 0.01 at 10 minutes).

The Ald (10⁻⁷ mol/L)–induced ERK phosphorylation was abolished with MR blockade (Figure 4D), whereas EGF (10 ng/mL)–induced ERK phosphorylation was not influenced by Spi (Figure 4E). Spi did not inhibit EGF-induced ROS production (data not shown).

Ang II and Ald Signaling Is Mediated Through the EGFR
We preincubated VSMCs with increasing concentrations (10, 100, and 300 nmol/L) of AG 1478, a specific EGFR blocker. We then stimulated the cells with Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and a combination of both compounds. ERK phosphorylation was diminished dose-dependently by blocking the EGFR in all protocols (Figure 5, A through C).

Discussion
We found that Ang II and Ald both induced ERK 1/2 and JNK phosphorylation in VSMCs and that the agonists were additive. The stimulation was dependent on ROS generation, because GSH and Tiron strongly attenuated the phosphorylation. Spi did not inhibit early NADPH oxidase–dependent ROS generation. Instead, Spi affected the later phase of ROS.

Figure 2. A, Ang II (10⁻⁷ mol/L) stimulated ERK 1/2 phosphorylation in VSMCs at 2 minutes (n=4). B, Ald (10⁻⁷ mol/L) stimulated ERK 1/2 phosphorylation at 10 minutes (n=4). C, At 2 minutes, effects of Ang II and Ald were potentiated (*P<0.01; n=6). *P<0.05 Ang II vs Ang II+Ald. D, MAP kinase–induced Elk-1 phosphorylation was enhanced in VSMCs treated with Ang II and Ald after 2 minutes. E, Immunofluorescence and confocal microscopy confirmed potentiating effect of Ald on Ang II–induced ERK 1/2 phosphorylation.
generation, phosphorylation of the EGFR, and ERK 1/2 after Ang II stimulation. We then tested the notion that Ang II– and Ald-induced phosphorylation of ERK 1/2 was dependent on the EGFR. A specific EGFR blocker inhibited both the Ang II– and Ald-induced signaling events. We believe that these data are relevant to our in vivo findings that MR blockade with Epl reduced ERK 1/2 phosphorylation in dTGR vessels and greatly ameliorated Ang II–induced end-organ damage.

The MR is expressed not only in the cortical collecting duct but also in many other tissues, including the heart. Northern blotting, RNAse protection assay, RT-PCR, in situ hybridization, immunohistochemistry, and Ald binding studies have been performed in cardiac tissue. However, precise cellular localization studies have not been entirely satisfactory. Endothelial cells, cardiac fibroblasts, VSMCs, and cardiomyocytes have all been implicated in terms of MR expression. The MR can be occupied not only by Ald but also by glucocorticoids. As a matter of fact, the MR may dimerize with the glucocorticoid receptor.

Brilla et al. and Young et al. used different rat models and found that increased circulating Ald levels resulted in cardiac fibrosis. The DOCA-salt model results led to the suggestion that mineralocorticoid-mediated sodium entry into cardiac cells might be responsible. Further support came from the finding that Spi ameliorated the effects. Ang II regulates cardiac Ald production. Several studies were conducted to address the possibility that Ang II was responsible for cardiac fibrosis rather than Ald. Rocha et al. showed...
Figure 4. A, Spi (preincubation for 30 minutes) reduced Ang II–induced ERK 1/2 phosphorylation and EGFR phosphorylation at 10 minutes ($P<0.01$; $n=4$ each) but not at 2 minutes. B, Same effects were documented with immunofluorescence and confocal microscopy. C, Spi (preincubation for 30 minutes) reduces Ang II–induced generation of ROS ($P<0.05$ from 5 minutes; $n=5$ each). D, Spi (preincubation for 30 minutes) abolished Ald-induced ERK 1/2 phosphorylation ($P<0.01$; $n=7$ each). E, Spi (preincubation for 30 minutes) did not influence EGF-induced ERK 1/2 phosphorylation ($P=0.88$; $n=3$ each).
that Ald infusion stimulates cardiac fibrosis in the rat. They
suppressed Ang II production simultaneously with an ACE
inhibitor. Benetos et al20 used a combined infusion of Spi and
an ACE inhibitor in spontaneously hypertensive rats. They
found that Spi reversed cardiac fibrosis. Earlier, we studied a
similar rat model using Spi.21 In that study, we also found that
Spi ameliorated cardiac hypertrophy and fibrosis, largely
independently of blood pressure. Taken together, these stud-
ies support the notion that Ald induces cardiac fibrosis
independently of other renin-angiotensin system components.

Ullian et al22 first suggested that Ald increases Ang II
receptor number, increases Ang II–stimulated inositol phos-
phate responses, and prevents the Ang II–induced downregu-
lation of Ang II receptors. This group also showed enhanced
phospholipase Cγ–dependent signaling when VSMCs were
preincubated with Ald for 24 hours before Ang II stimula-
tion.23 In contrast to our findings, transcriptional regulation
was involved in the studies by Ullian et al. We cannot exclude
the possibility that genomic and nongenomic effects might
have contributed to our in vivo observations. Nevertheless,
the short duration of our in vitro experiments, as well as our
results obtained with actinomycin D and cycloheximide,
support a nongenomic effect.

Nongenomic effects have been reported for other steroids.
Limbourg et al24 described a rapid and nontranscriptional
activation of eNOS by corticosteroids that is transmitted via
phosphatidylinositol 3-kinase and Akt. Nongenomic Ald-
related effects have also been described in humans.6 For
instance, Schmidt et al25 found that Ald, via nongenomic
mechanisms, has diverse effects on the cardiovascular system
that depend on the preexisting adrenergic state. Data from the
rat remnant kidney model also support the idea that chronic
Ald-related effects may include a nongenomic component.
Greene et al26 studied 5/6 nephrectomy remnant kidney rats
given AT1 receptor blockers with ACE inhibitors and com-
pared them with remnant kidney rats given these drugs along
with Ald. In the former group, Ang II–related effects were
blocked, and the rats were protected. In the latter group, given
Ald, the effects were not ameliorated. This group resembled
the no-treatment remnant kidney group. In their study, MR
blockade did not reverse the effects of Ald, suggesting that
MR-independent effects were present. The effects that we

Figure 5. A and B, Ang II and Ald-induced ERK 1/2 phosphorylation was reduced by EGFR blocker AG 1478 dose-dependently (preincubation for 30 minutes). C, A similar inhibitory effect of AG1478 was obtained on Ang II–Ald–induced ERK 1/2 phosphorylation, indicating that combination was also EGFR-dependent (n=4 each).
trafficking might be needed for EGFR internalization and tyrosine kinase signaling. The cytosolic tyrosine kinase c-src requires a functioning EGFR for Ang II–NADPH oxidase activation and ROS generation.30,33 Also, aldosterone also participated in MAP kinase family that are also activated by ROS. The ERK pathway also modulates the expression of genes via phosphorylation of the transcription factor Elk-1, which controls the production of the c-Fos transcription factor. Nevertheless, not all Ang II–induced MAP kinase activation is under control of the EGFR.34 Our data show that not only Ang II but also aldosterone participate in both JNK and ERK signaling. They suggest that blockade of both the AT1 and the MR receptor may be necessary to accrue maximal effects in terms of vascular protection.

Acknowledgments

These studies were supported by grants-in-aid from the Klinisch-Pharmakologischer Verband Berlin-Brandenburg, the Deutsche Forschungsgemeinschaft (to Dr Muller), the Nationales Genomnetzwerk (NGNF), and Pharmacia Inc, USA. We thank A. Bussjahn for help with the statistical analysis and M. Kamimura, M. Köhler, C. Lipka, G. N’Diaye, P. Quass, and M. Schmidt for their excellent technical assistance.

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Circulation. 2004;109:2792-2800; originally published online May 24, 2004; doi: 10.1161/01.CIR.0000131860.80444.AB
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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